THE SYNTHESIS AND ENZYAMTIC CHARACTERIZATION OF 6-AMINO-FMN WITH BLUB; THE LAST UNSOLVED BIOSYNTHETIC MODULE OF VITAMIN B_{12}

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Synthesis and Enzymatic Characterization of 6-amino-FMN with *BluB*; The Last Unsolved Biosynthetic Module of Vitamin B_{12}

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The last unsolved module for the biosynthetic pathway of vitamin B_{12} is the biosynthesis of the lower axial ligand, dimethylbenzimidazole (DMB). Recently, the oxygen-dependent enzyme, BluB, has been shown to catalyze the oxidative fragmentation of coenzyme B_2, FMN, and produces dimethylbenzimidazole (DMB), erythrose-4-phosphate, and alloxan. BluB is the first enzyme shown to utilize FMN as a substrate rather than as a coenzyme. Our goal is to elucidate the mechanism by which BluB catalyzes the oxidative fragmentation of FMN with molecular oxygen. Previous studies, done by our group, with numerous substrate analogues have shown no enzymatic activity with BluB and conclude that the active site of BluB is highly selective for the native substrate. In this study, the synthesis of 6-amino-FMN and its activity with BluB are described. LCMS analysis of the enzymatic assays confirmed the production of 4-amino-DMB. This discovery suggests that active site of BluB tolerates substrate analogues with substituents at the 6-position and additional 6-substituted substrate analogues can be designed to provide mechanistic insight.

ACKNOWLEDGEMENTS

I would really like to thank Dr. Tadhg P. Begley for providing me the opportunity to be a member of his lab. I am forever thankful for his patience and guidance. I would also like to thank the all the members in the Begley group. I am thankful for the help and support they provided me. Finally, I would like to thank my family, specifically, my mom. Her support allowed me to develop a passion for chemistry.

CHAPTER I

INTRODUCTION

Cobalamins are a family of natural products that were the first identified biomolecules to possess a carbon-metal bond. Cobalamins are organometallic complexes that consist of cobalt metal in an octahedral geometry and chelated by a cobamide structure. A cobamide is heterocyclic structure that consists of a tetra-pyrrole ring, known as a corrin ring and a nucleotide loop.[1] The corrin ring coordinates equatorially and the nucleotide loop serves as the lower axial ligand.[1] Cobalamins are distinct class of cobamides in that their nucleotide loop consists of a benzimidazole base, usually dimethylbenzimidazole (DMB).[2] The upper axial coordination site is the reactive site for the B_{12} complex and the specific enzyme the B_{12} coenzyme is bound to dictates the ligand coordinated at this site.[1] Vitamin B_{12} denotes a cyano-substituent as the upper axial ligand **(Figure 1)**.

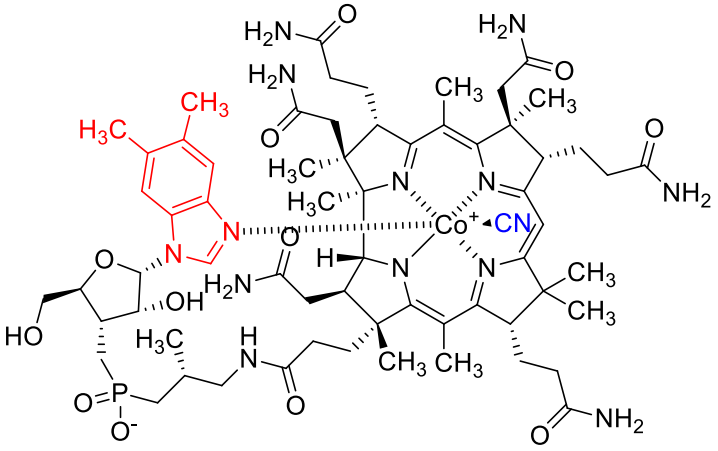


Figure 1. The structure of vitamin B_{12} (cyanocobalamin). DMB is highlighted in red and the cyano-ligand in blue.

In humans, the 2 biologically active forms of cobalamin have a methyl group (Methylcobalamin) and the 5'-carbon of adenosine (Adenosylcobalamin) as the upper axial ligand.[3] Cobalamin dependent enzymes catalyze intramolecular rearrangements, intermolecular methylations, and the reduction of NTPs.[1] In humans, methionine synthase and (R)–methylmalonyl–CoA mutase are the only enzymes that require coenzyme B_{12} in order to perform their catalytic functions.[1] methionine synthase utilizes methylcobalamin, along with 5-methylfolate as coenzymes to catalyze the biosynthesis of methionine from homocysteine.[1,4] (R)–methylmalonyl–CoA mutase utilizes adenosylcobalamin as its coenzyme. This enzyme is utilized in fatty-acid, amino acid, and carbohydrate metabolism where it catalyzes the intramolecular radical rearrangement of (R)–Methylmalonyl–CoA to give Succinyl-CoA.[1,4] The reactions catalyzed by methionine synthase and (R)-methylmalonyl-CoA mutase are shown below **(Figure 2).**

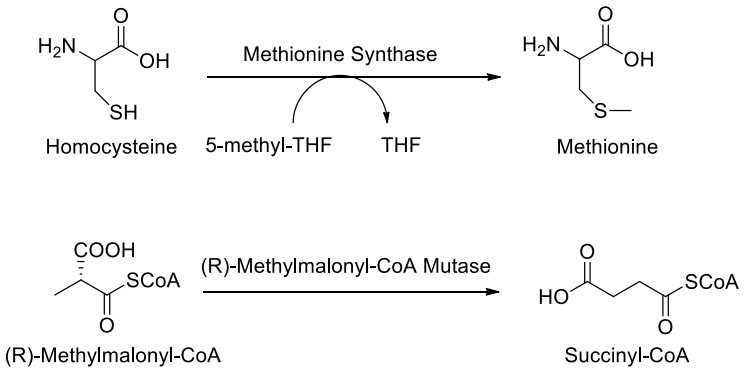


Figure 2. The reactions catalyzed by the 2 B_{12}-dependent enzymes in humans: methionine synthase and (R)–methylmalonyl–CoA mutase.[1]

The de novo biosynthetic pathway of B_{12} requires ~ 30 enzyme-mediated steps and is restricted to a handful of prokaryotes.[3] Complete characterization of B_{12}'s biosynthetic pathway is nearly done where the biosynthesis of the nitrogenous base, DMB, of the lower nucleotide loop is the

last uncharacterized biosynthetic module. Recently, the BluB protein has demonstrated DMB synthase catalytic activity via a novel oxidative fragmentation of flavin mononucleotide (FMN) in the presence of O_2 into DMB, alloxan, and erythrose-4-phosphate **(Figure 3).**[2]

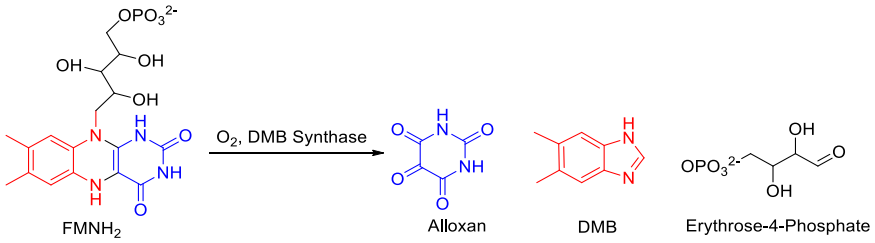


Figure 3. The oxygen-dependent reaction catalyzed by DMB synthase.

BluB is the first known enzyme capable of fragmenting the isoalloxazine ring of FMN into DMB.[2] The active site of BluB resembles the active site of enzymes within the oxidoreductases and mono-oxygenases family.[5] This suggests that subtle structural differences in the active site deviate BluB's catalytic activity from an oxidoreductase to a DMB synthase.[5]

In this study, substrate analogues are designed and synthesized in order to probe the mechanism of the DMB synthase. Substrate analogues are designed to resemble the native substrate enough for substrate recognition by the active site but differ from the native substrate enough to trap an intermediate in the mechanism or lead to the formation of a shun product. The ease of conducting the synthetic scheme to obtain the substrate analogue is another important factor to consider when designing substrate analogues. Isotopic labeling studies of the BluB enzymatic production of DMB have lead to the current mechanism proposal **(Figure 4).**

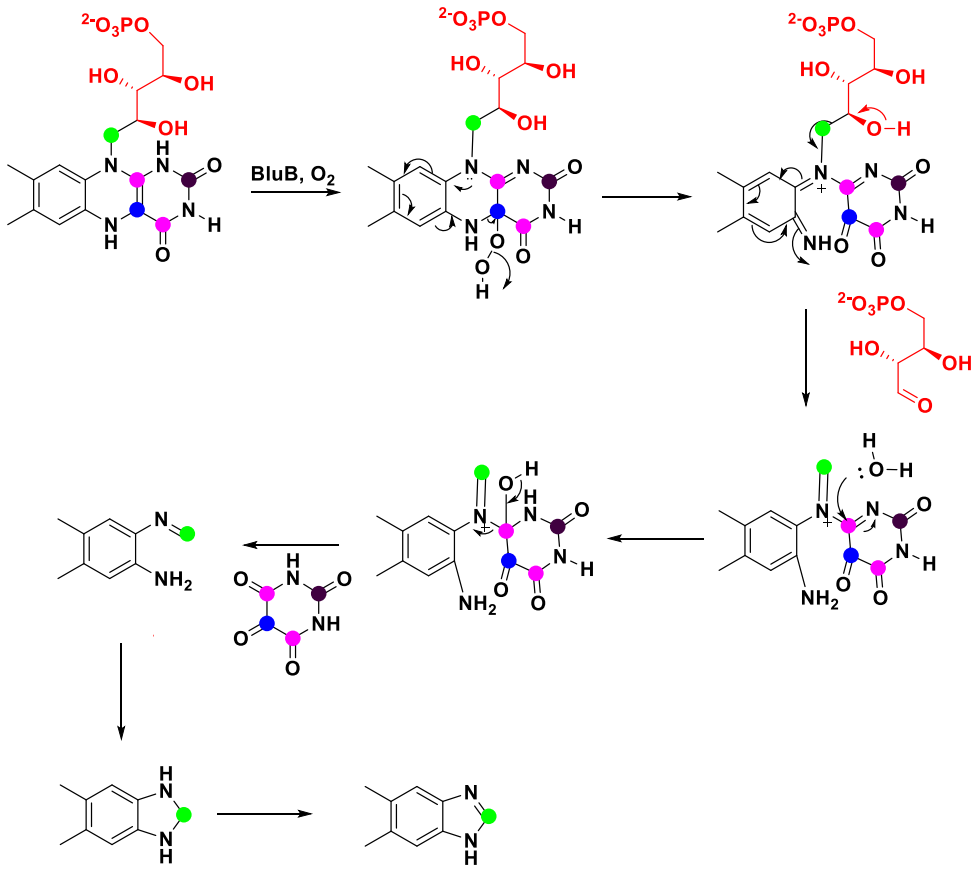


Figure 4. Enzymatic studies conducted with FMN isotopologues, where each colored carbon depicts an isotopologue with ^{13}C at that designated position.

In a previous study, we examined if substrate analogues with a truncated ribityl tail displayed enzymatic activity. The derivative of riboflavin, hydroxyethylflavin (HEF), was synthesized was shown to be enzymatically inactive with BluB when introduced with either glycerol-3-phosphate (G3P) or glycol-2-phosphate (G2P) as co-substrates.

The X-Ray crystal structure of BluB with bound FMN was utilized as a guide to design additional substrate analogues. From the crystal structure, it was concluded that the 6- and 9-positions of the isoalloxazine ring were not sterically crowded by amino acid residues **(Figure 5).** Thus, we hypothesize that substrate analogues with substituents at the 6- and 9- position of FMN will display enzymatic activity. The substrate analogue 6-nitro-FMN was initially chosen to test this hypothesis.

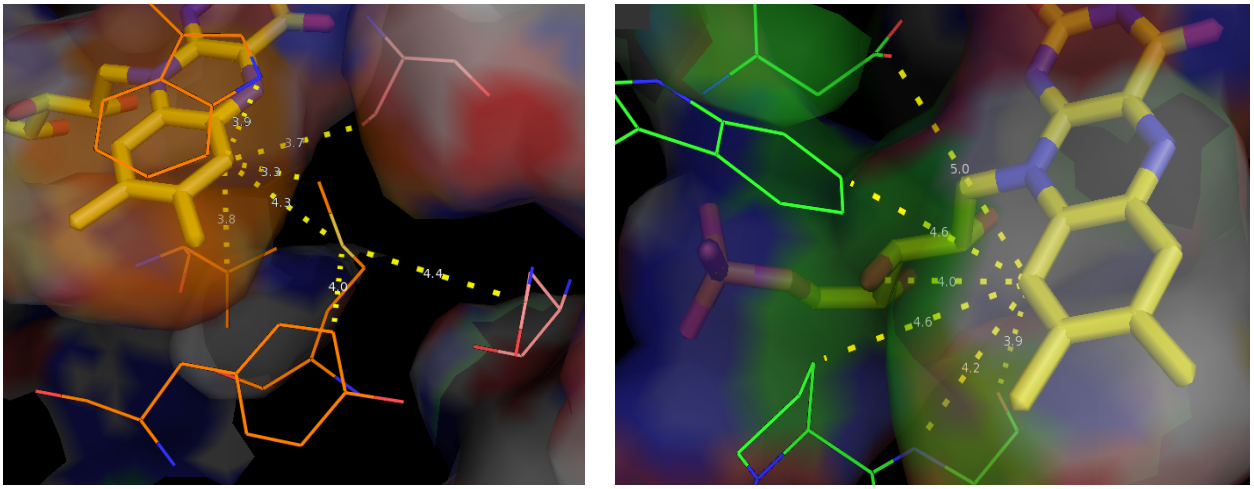
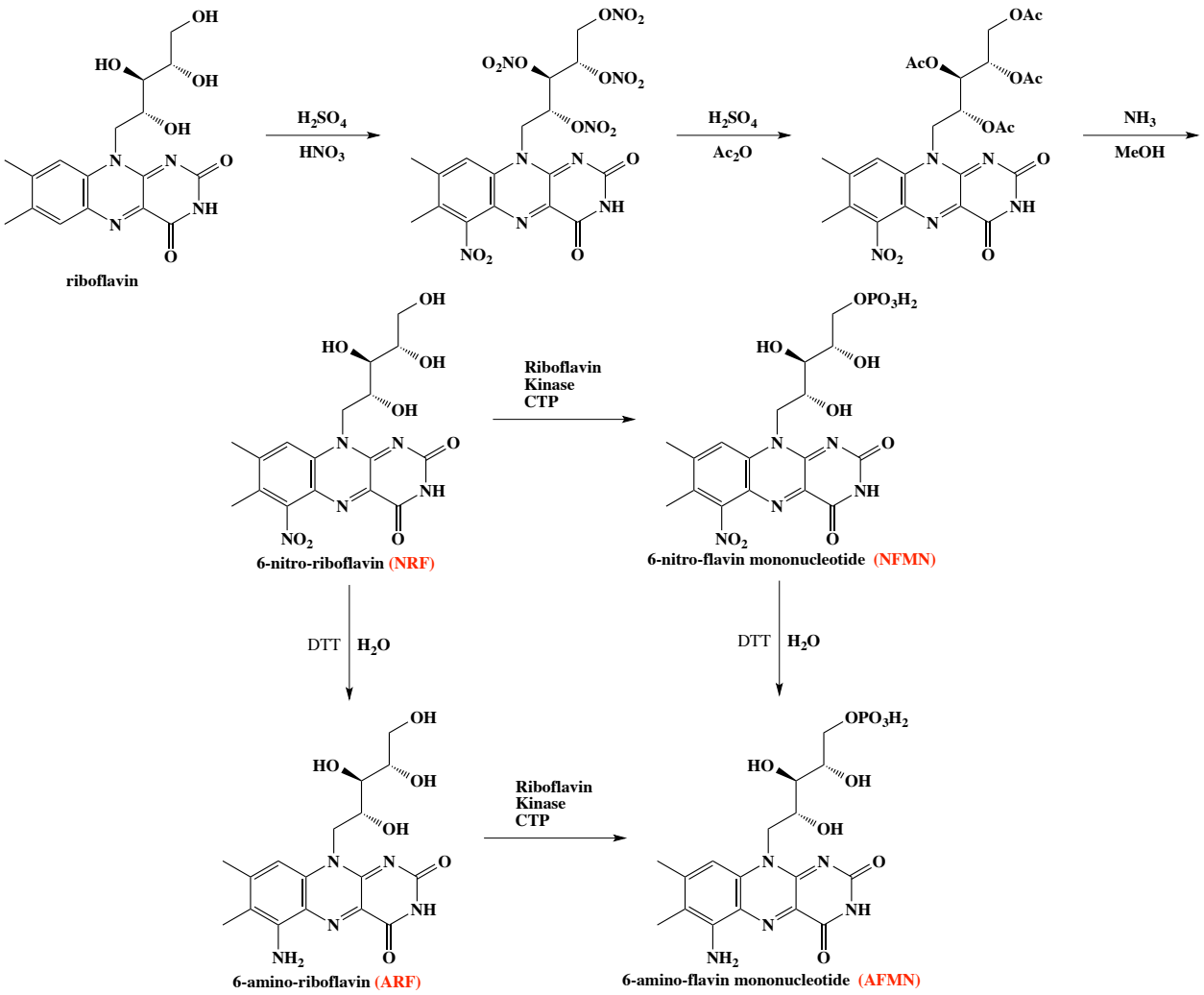


Figure 5. The active site structure of BluB with the distance between the C6 (left) and C9 (right) FMN carbon to the nearest protein residues.



Scheme 1. Synthetic Methodology to synthesize 6-nitro-riboflavn and 6-amino-riboflavin The enzymatic methodology utilized to obtain 6-amino-FMN.

To synthesize 6-nitro-FMN, 6-nitroriboflavin (NRF) was synthetically obtained and enzymatically phosphorylated by riboflavin kinase, shown in **scheme 1**. This analogue was originally chosen as the target molecule to probe BluB for its electron withdrawing properties. The π and inductive electron withdrawing character of the nitro- substituent will perturb the electron density of the isoalloxazine ring. This perturbation could potentially alter an enzymatic

step and provide an intermediate or lead to the production of a shun product. However, it became apparent that the 6-nitro-riboflavin species was photochemical unstable and slowly undergoes spontaneous photoreduction to 6-amino-riboflavin (ARF). The amino species was also obtained with the addition of several reducing agents. This was observed with NADH and sodium hyposulfite as the reducing agent **(Figure 6)**. The facile chemical and photoreduction of NRF prevented the characterization of BluB with pure NRF and ARF was instead chosen as the substrate analogue for enzymatic characterization with BluB. The 6-amino-flavin derivatives were synthetically obtained via **scheme 1**. The 6-amino derivative will provide insight to the active site's compatibility to 6-substituted analogues. If ARF is enzymatically active, further 6-substituted substrate analogues will be designed, such as 6-cyano-FMN (CFMN), from NRF.

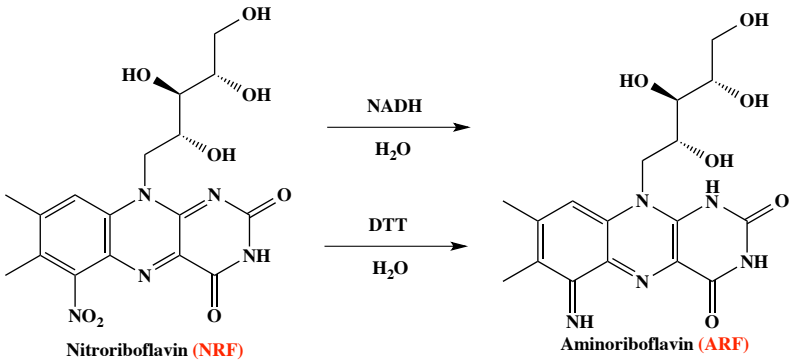


Figure 6. Synthetic methods utilized for the reduction of 6-nitro-riboflavin to 6-amino-riboflavin.

CHAPTER II

METHODS

All commercially available reagents were purchased from Sigma-Aldrich and were used without further purification. Riboflavin and DMB were purchased from Sigma-Aldrich. All reactions were performed within a fume hood. A heating mantle was used when a reaction needed to be heated. Removal of volatile solvents was accomplished via a rotary evaporator. The characterization of all substrate analogues was conducted with a 400 MHz Bruker Nuclear Magnetic Resonance (NMR) spectrometer, a Qstar Mass Spectrometry (MS), and a Liquid Chromatography Mass Spectrometer (LCMS). The biosynthesis and purification of DMB synthase was worked up from the vector containing the BluB gene. The biosynthesis of the DMB synthase protein required the purification of the BluB plasmid, the transformation of BluB into the E. Coli BL-21 strain, the overexpression of large cultures, and purification by Ni column chromatography. The enzyme was stored in an -80$^{\circ C}$ freezer until needed for enzymatic assays. All of the preceding procedures were conducted in LB broth media or agar containing 25 μg/ml of ampicillin (Amp) unless stated otherwise. Enzymatic assays where prepared in a glove box and analyzed through HPLC.

Synthesis

6-(2',3',4',5')-pentanitro-riboflavin

Riboflavin (1g, 2.66 mmol) was dissolved in fuming nitric acid (10 mL). Sulfuric acid (1mL) was slowly dropwise added to the reaction mixture at rt. The reaction mixture was stirred for 20 hours then poured into 20-30 mL of ice water and allowed to sit in the cold room overnight. The

precipitate was vacuum filtered, washed with water until the pH was neutral, and dried. A dark orange solid (1.245 g, 2.07 mmol, 78% yield) was obtained.

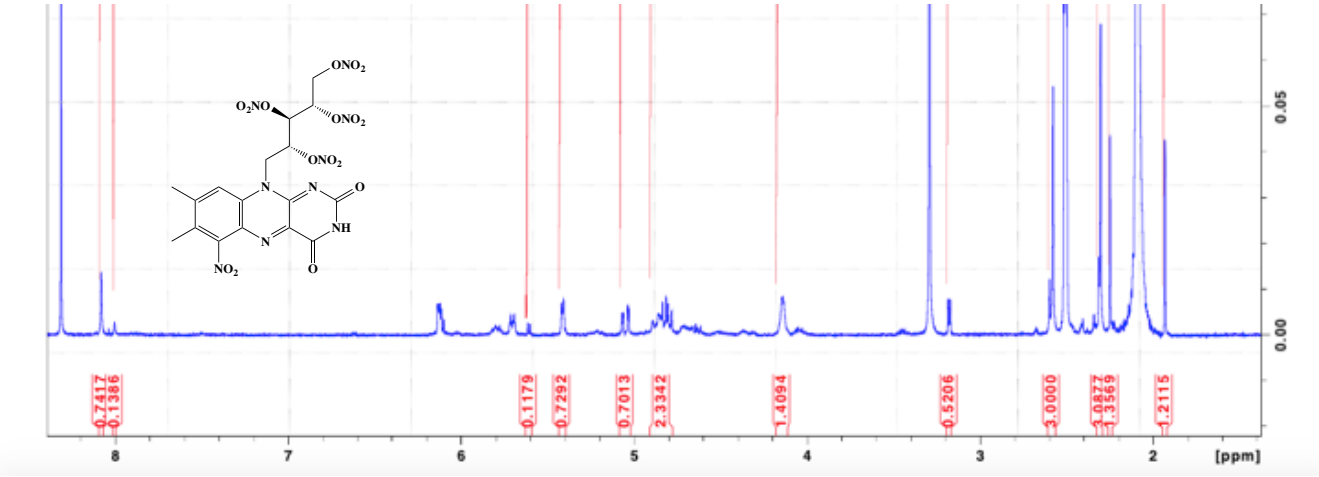


Figure 7. 6-(2',3',4',5')-pentanitroriboflavin ^{1}H spectrum: (400 MHz, DMSO) δ 8.32 (s, chloroform), 8.08 (s, 1H), 8.00 (s, .13H) 6.12 (s, 1H), 5.80 (m, .5H), 5.42 (d, 1H), 5.05 (d,1H), 4.84 (m, 2H), 4.67 (m, 1H), 4.15 (s, 1H), 3.29 (s, water), 3.18 (d, .5H), 2.59 (s, 3H), 2.51 (q, DMSO), 2.31 (s, 3H), 2.25 (s, 1H), 2.08 (s, acetone), 1.93 ppm (s, 1H).

(2',3',4',5')-tetraacetyl-6-nitro-riboflavin

6-(2',3',4',5')-pentanitroriboflavin (1 g, 1.66 mmol) was added to dry acetic anhydride (10 mL). The reaction mixture was placed on ice and then had sulfuric acid (1mL) added dropwise. The reaction mixture was stirred for 2 hours at 50-60$^{\circ C}$. The reaction mixture was allowed to cool to rt and diluted with cold ethanol (75 mL). The reaction mixture was allowed to sit for 5 minutes followed by the addition of ice water (75 mL). The reaction mixture was concentrated *in vacuo* to approximately 30 mL and then cooled on ice. The precipitate was collected by vacuum filtration and the mother liquor was diluted with ethanol (15 mL) and again concentrated *in*

vacuo and placed on ice. The precipitate was again vacuum-filtered and added to the previous precipitate. The obtained oil diluted with water and extracted with ethyl acetate. The organic layer was dried with $MgSO_4$ and concentrated *in vacuo*. A yellow/orange (421 mg, .715 mmol, 43% yield) crude solid was obtained. Column Purification was conducted with 1:4 acetonitrile: ethyl acetate.

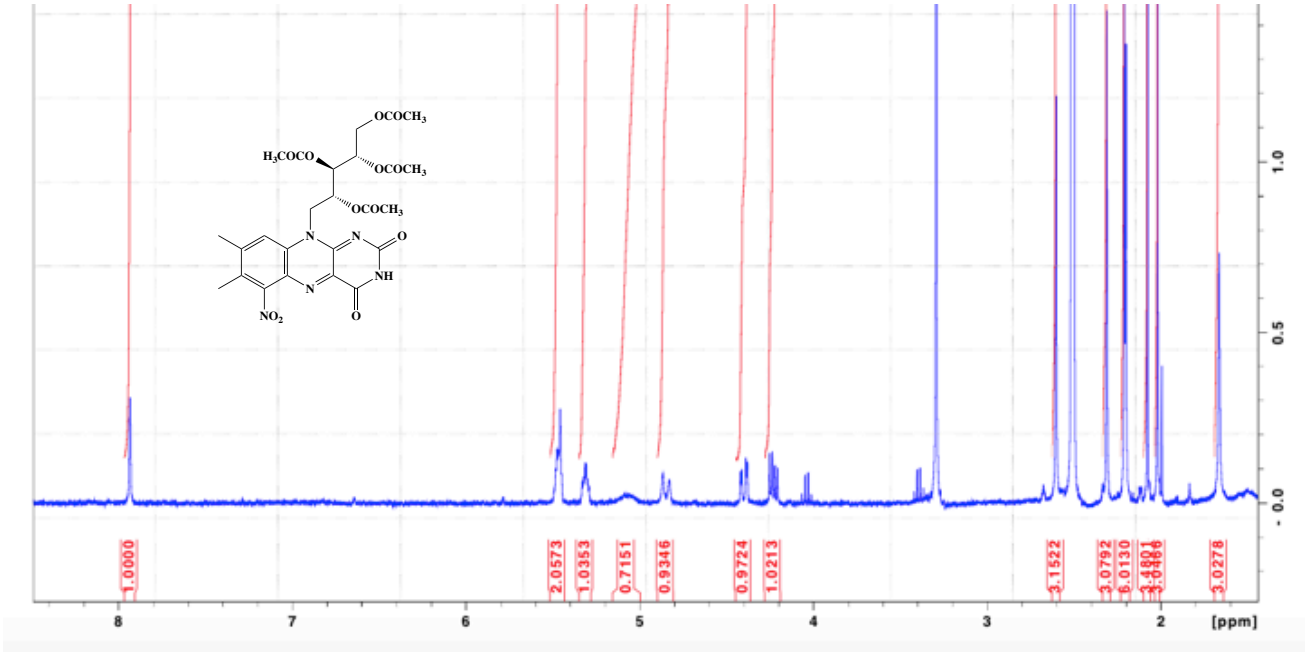


Figure 8. (2',3',4',5')-tetraacetyl-6-nitroriboflavin ^{1}H spectrum: (400 MHz, DMSO) δ 7.94 (s, 1H), 5.48 (s, 2H), 5.32 (t, 1H), 5.07 (m, 1H), 4.85 (d,1H), 4.41 (d, 1H), 4.24 (m, 1H), 3.30 (s, water), 2.60 (s, 3H), 2.51 (q, DMSO), 2.31 (s, 3H), 2.21 (s, 6H), 2.07 ppm (s, acetone), 2.02 (s, 3H), 1.67 ppm (s, 3H).

6-nitroriboflavin[4]

(2',3',4',5')-tetraacetyl-6-nitroriboflavin (70 mg, 119 μmol) was dissolved in methanolic ammonia (10 mL). The solution was left to stir overnight at rt. The reaction mixture was concentrated *in vacuo*, had methanol (10 mL) added and again concentrated *in vacuo*. The crude was suspended in water and lyophilized overnight to provide a red-orange crude solid (40 mg, 95.0μmol, 80% yield).

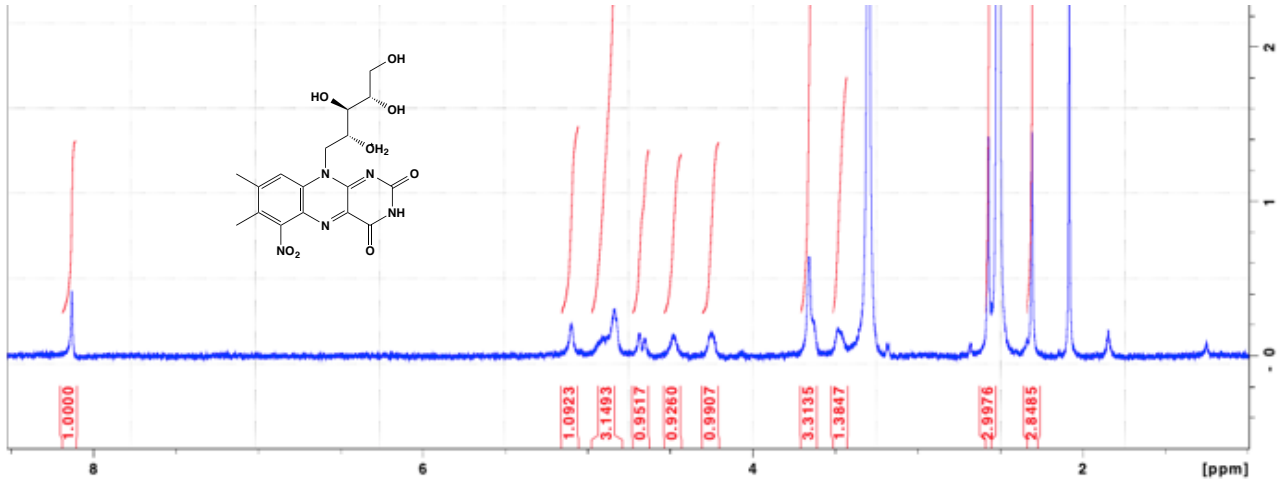


Figure 9. **6-Nitro-riboflavin ^{1}H spectrum: (400 MHz, DMSO) δ** 8.15 (s, 1H), 5.11 (s, 1H), 4.89 (m, 3H), 4.68 (d, 1H), 4.49 (s,1H), 4.26 (m, 1H), 3.66 (s, 3H), 3.47 (m, 1H), 3.33 (s, water), 2.57 (s, 3H), 2.50 (q, DMSO), 2.30 (s, 3H), 2.08 ppm (s, acetone).

6-aminoriboflavin

Was prepared *in situ* by the addition of sodium hyposulfite to aqueous 6-nitroriboflavin. The reaction was monitored by the color of the solution. The brown-green solution obtained was lyophilized to provide a dark-green solid. ^{1}H NMR was not taken.

Molecular Genetics

Plasmid Extraction

Purification of the plasmid with the BluB gene was purified from the E. Coli DH5α strain. The strain was streaked onto a 25 μg/ml of ampicillin (Amp) LB agar plate and incubated at 37$^{\circ C}$ for 12 hours. From this plate, a single colony was inoculated with 5 ml of 25 μg/ml Amp LB media and placed overnight in a shaker at 37$^{\circ C}$ and 220 rpm. This culture was centrifuged for 3 minutes at 10,000 rpm and decanted. The pellet was resuspended in 250 μl of P1 buffer. Next, 350 μl of P2 buffer was added and inverted. Upon the appearance of a blue color, 350 μl of N3 buffer was added and inverted until the blue color was gone. The sample was centrifuged for 10 minutes at 13000 rpm. The supernatant was decanted to a QIA prep spin column where it was centrifuged for a minute. The QIA prep spin column was then washed, centrifuged for a minute at 13000 rpm, and had the flow through discarded once with 500 μl of PB buffer and twice with 750 μl of PE buffer. The QIA prep spin column was centrifuged for an additional 2 minutes. Finally, to elute the plasmid, 50 μl of EB buffer was added, the QIA prep spin column was transferred to an eppendorf tube, and let sit for 1 minute. The QIA prep spin column was then centrifuged and the eluent was stored at 20$^{\circ C}$.

Plasmid Transformation

The transformation of the BluB plasmid into BL21 competent cells was conducted by electroporation. The transfer of 100 μl of compotent cells and of 1 μl of the BluB plasmid into an electroporation cuvette was performed on ice and in the presence of an open flame. The electroporation tube was quickly dried, shocked with a 25 kV electrical pulse, and mixed with 1 mL of media. The culture was then incubated in a shaker at 37°C for 90 minutes. Next, the culture was centrifuged and the media was removed until 100 μl of media with the cell pellet was all that remained. The cell pellet was resuspended and spread on an agar plate and incubated at 37°C for 12 hours.

Protein Biochemistry

Protein overexpression

The overexpression of BluB was performed by inoculation of a single colony into 10 ml of media and grown overnight at 37°C. Approximately 5 ml of this culture was added to each of the two 3 L flasks containing 1.5 L of media. The culture was grown at 37°C until its optical density at 600 nm reached 0.600. The BluB gene was induced by the addition of .1mM IPTG while lowering the temperature to 30°C. The cultures were incubated for an additional 16-18 hours before harvesting the cells and stored at -80°C.

Protein Prep

Purification of BluB from the cell pellet was conducted via affinity chromatography. A Ni column was utilized with all solutions kept under ice at 4°C. The cell pellet was thawed and suspended in 20 mL of lysis buffer containing 2 mM DTT and 5 mg of lysozyme for 25 minutes.

The cells were placed under sonication for five periods of 20 seconds each. The cell lysate was centrifuged for 30 minutes at 17000 rpm and 4°C then passed through a 50K syringe filter. To prepare the Ni column for the cell extract, the Ni column was washed with 5 column volumes of water and then 3 column volumes of lysis buffer. The supernatant was loaded onto the column and the flow through was collected and stored on ice. The column was washed with a gradient of imidazole elution buffer. The gradient consisted of 15 ml fractions of 20, 50, 100, 150, 200, and 250 mM imidazole elution buffer. A fraction was collected for each imidazole concentration and stored on ice. Each collected fraction was examined via an SDS-PAGE to determine the fraction that possessed the pure protein. The fraction(s) with purified protein were combined and centrifuged in a 10K filter tube until a total volume of 3 mL was obtained. The 3 mL of purified protein was loaded onto a desalting column and eluted with 3 mL of desalting buffer, which possessed 2 mM DTT. The purified protein was distributed into 30, 100 μL aliquots, which were flash frozen in liquid nitrogen and stored at -80°C. Protein concentration was determined via absorbance with a nano-drop spectrometer at 260 nm.

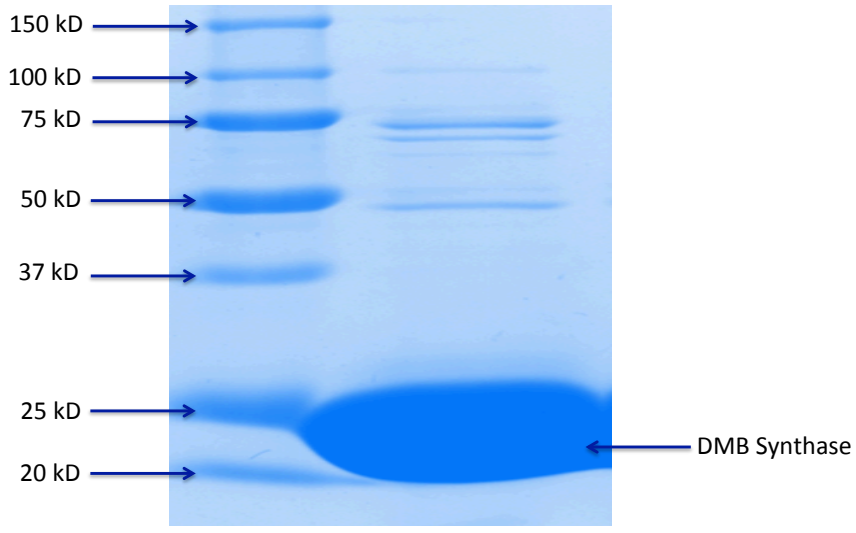


Figure 10. The SDS-PAGE from the purification of DMB synthase via a Ni(II) column.

Enzymatic Assays and HPLC Characterization

All of the starting materials were prepared in phosphate buffer at a pH of 7.5. Prior to conducting the enzymatic assays, the stocks of the starting materials had to be made. The NRF stock solution consisted of the extent of dissolution of NRF (1 mg) in 2 mL phosphate buffer. Two stocks were made where a small arbitrary amount of sodium hyposulfite was added to 1 of the stocks until the solution appeared faint clear yellow. This solution was exposed to O_2 and shaken until it became a sage green-brown color. The UV-Vis spectrum of this solution was taken for visual confirmation of ARF spectrum, **(Figure 14)**. The RibK stock was acquired from a lab member with an unknown concentration. For each RibK assay, 10 μL of the RibK enzyme was added and contained 1 mM CTP. For the BluB assays, the concentration of NADH, FRE, BluB, and FMN were 2 mM, 200 nM, 100 μM, and 30 μM respectively. The concentration of AFMN was unknown because the percent conversion of ARF to AFMN was unknown. These assays were typically conducted with a final volume of 100 or 120 μL. HPLC buffer utilized was 10 mM ammonium acetate and the organic solvent was methanol.

CHAPTER III

RESULTS

Synthesis

The synthesis of NRF was a difficult process due to its photosensitivity. It required multiple attempts of each reaction in **scheme 1** and provided a total yield of 3.6%. Each reaction was monitored via TLC characterized via ^{1}H NMR and MS to confirm the product. To synthesize NRF, riboflavin was first nitrated with a mixture of nitric acid and sulfuric acid. This reaction was unsuccessfully attempted a few times, in different conditions, with concentrated nitric acid. Nitration did not occur with concentrated nitric acid and required fuming nitric acid, which was purchased from Sigma-Aldrich. This was confirmed via the presence of the 2 aromatic protons in the ^{1}H NMR spectrum of the crude product. With fuming nitric acid, the best yields were obtained with 10:1 ratio of fuming nitric acid to concentrated sulfuric acid with 1 g of riboflavin was stirred at rt for 20 hours. TLC analysis was optimized with 1:4 MeOH:EtAc as the mobile phase and clearly showed the presence of multiple fluorescent bands under UV light. Column purification was initially but identification of the desired product was too difficult, thus, it was decided to proceed to step 2 with the crude mixture.

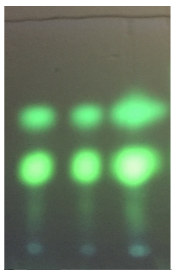


Figure 11. TLC characterization of TANRF and TARF. TANRF $R_f = 0.6$, TARF $R_f = 0.4$

Reaction 2 was the acetylation of 6-(2',3',4',5')-pentanitro-riboflavin with acetic anhydride under acidic conditions. The reaction was performed as described in the given procedure. Precipitation of the product was the most difficult part of the procedure. The original procedure suggests to vacuum filter the precipitate after the addition of water. However, when the reaction was worked up in accord with the procedure, no precipitate was initially observed. The work up was modified for product precipitate. To precipitate (2',3',4',5')-tetraacetyl-6-nitro-riboflavin, the reaction mixture was diluted with additional ethanol and water. Recrystallization in ethanol was also attempted as well as other solvents. The reaction mixture was concentrated *in vacuo* to approximately 30 mL and cooled on ice. This technique triggered the formation of some precipitate, which was vacuum filtered after precipitation appeared to stop. After in vacuo concentration of the mother liquor, the addition of water to the deep red oil obtained sparked the appearance of a bright yellow precipitate. The precipitate was vacuum filtered and the mother liquor was extracted with ethyl acetate dried with $MgSO_4$, and oncentrated *in vacuo*. All fractions were combined, suspended in water and lyophilized to dryness to yield 421 mg of crude product. The mobile phase optimized for TLC characterization was 1:4 AcCN:EtAc with 1 drop of glacial acetic acid added. The 2 bands correspond to (2',3',4',5')-tetraacetyl-6-nitroriboflavin (TANRF) at R_f = .6 and (2',3',4',5')-tetraacetyl-riboflavin (TARF) at R_f = .35**,** (Figure11)**.** Flash- column chromatography of 224 mg of crude (2',3',4',5')-tetraacetyl-6-nitroriboflavin provided(76 mg, 126μmol, 7.6% yield) pure (2',3',4',5')-tetraacetyl-6-nitroriboflavin, which appears as a bright yellow fine-powder.

The deacetylation of (2',3',4',5')-tetraacetyl-6-nitroriboflavin was accomplished with sufficient methanolic ammonia for dissolution. The reaction mixture was stirred overnight in the dark with

the round bottom wrapped in double layer aluminium foil. Work up was conducted as described above and crude NRF was stored in the dark at -20°C until needed for enzymatic assays. NRF was confirmed via ^{1}H NMR and UV-Vis spectroscopy, and LCMS characterization. The ^{1}H NMR spectrum of NRF was overlapped with the ^{1}H NMR spectrum of riboflavin to confirm the presence of the nitro substituent. The aromatic singlet of NRF should be downfield from the 2 aromatic singlets of riboflavin, spectrum provided in appendix A.

Enzymatic Assays

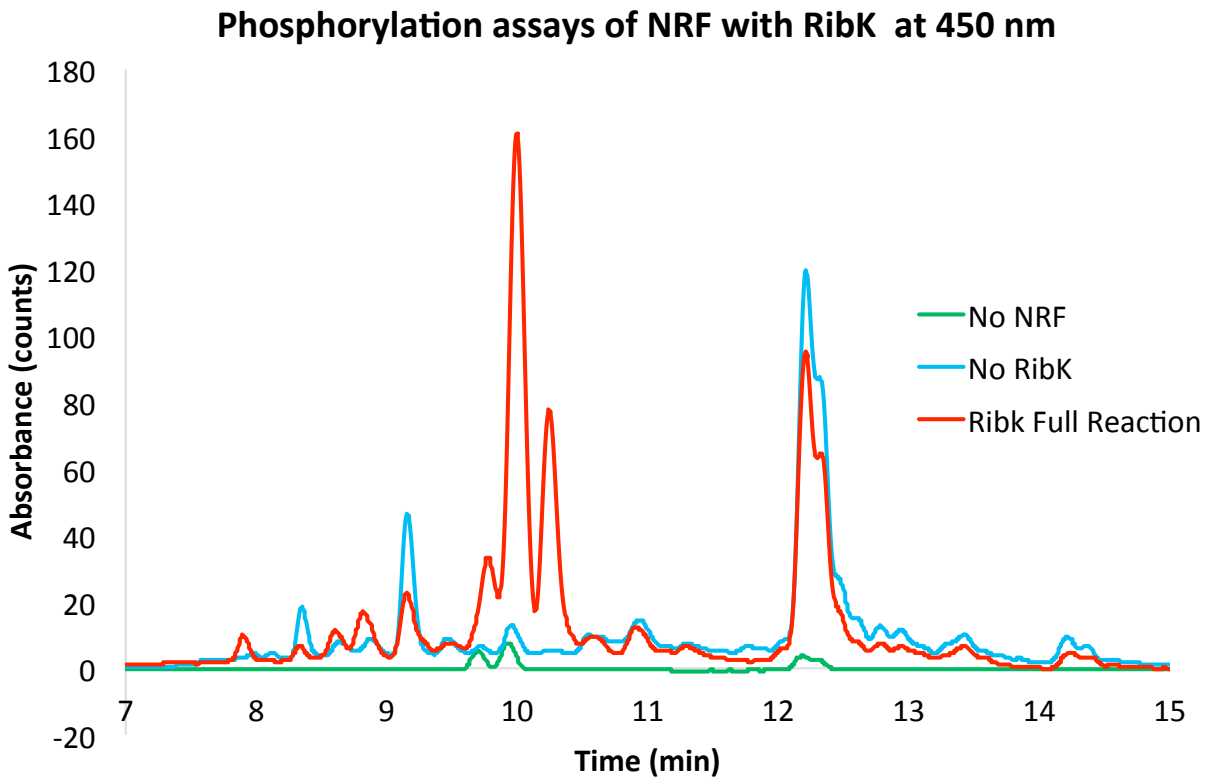


Figure 12. HPLC chromatograms of RibK assays at 450 nm. Retention times (t_r): NRF = 12.2 min, NFMN = 10.0 min, ARF = 12.3 min, AFMN =10.2 min.

Initially (before the spontaneous reduction of NRF was known), HPLC purification and phosphorylation of NRF was attempted with NRF. Purification required 20-40 HPLC runs due to its low solubility in MeOH and in water, where some runs consisted of NRF dissolved in millipure water, phosphate buffer, and MeOH. The solvent was varied to observe if solvent effects would perturb the replication of the acquired chromatograms. It was found that in MeOH the NRF chromatograms were slightly narrower compared to aqueous solutions (data not shown). Enzymatic phosphorylation of NRF with RibK was analyzed via HPLC **(Figure 12).** The peak at 10.0 min is present only in the RibK full reaction. In this assay, the NRF peak at 12.2 min is a visually smaller peak, however, not by much when compared to the relative size of the new NFMN peak at 10.0 min. Enzymatic assays with a specific batch of NRF synthesized were conducted several times over the course of time of a few weeks. All assays were replicated several times over the course of 3 weeks. When the replicated assays were compared, the temporal spontaneous conversion of NRF and NFMN to 2 analogues species became apparent. The 2 new species have identical UV-Vis spectra, which significantly differ from the characteristic flavin spectrum seen for FMN and NRF analogues **(Figure 14).** In **figure 12**, the shoulder present at 12.35 min is the analogues species formed from NRF and the peak at 10.2 min is the analogues species of NFMN. LCMS characterization of the RibK assays confirmed the 2 species to be ARF and AFMN with a 7.1 and 4.2 ppm error, respectfully **(Figure 13).**

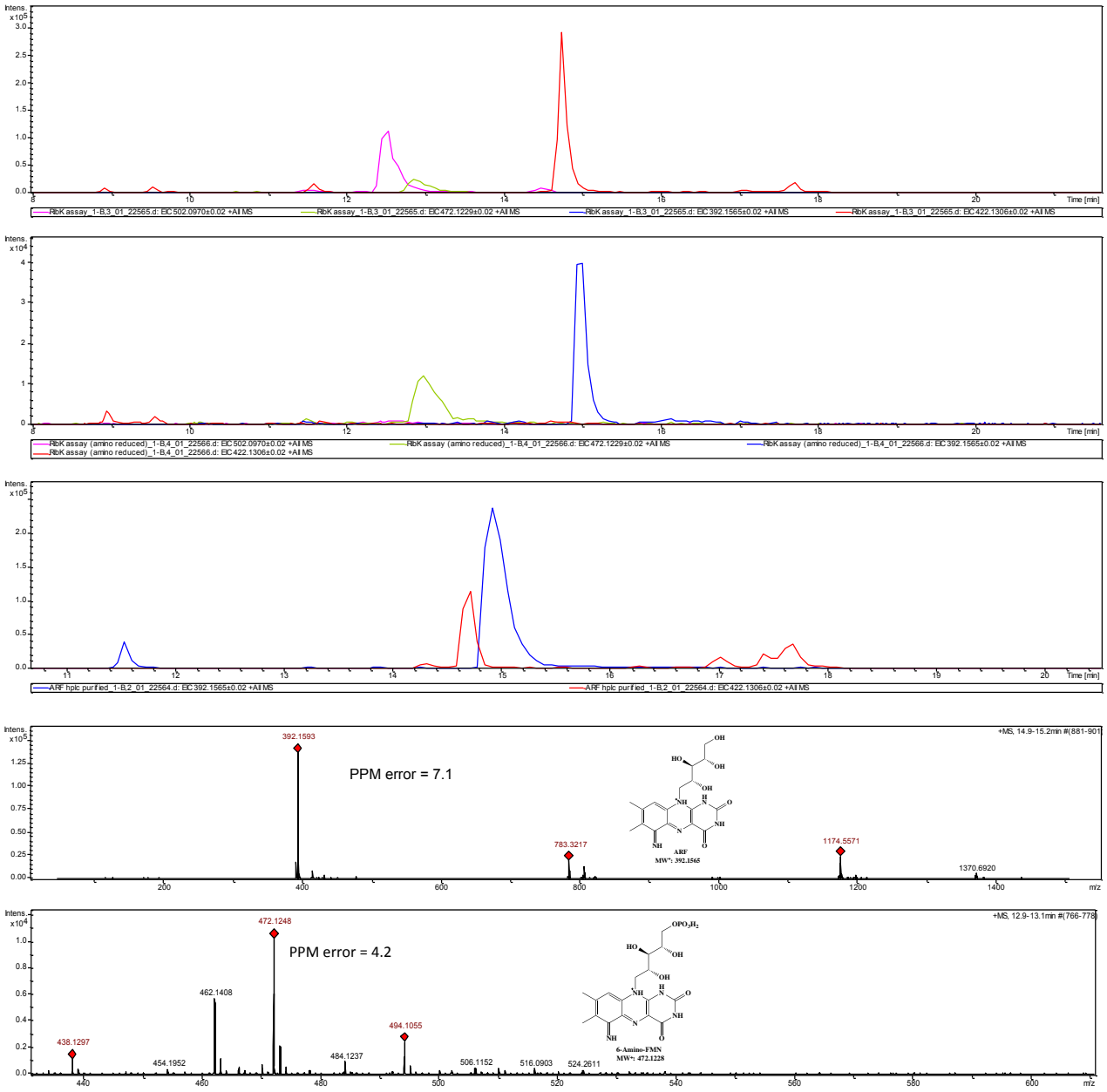


Figure 13. EIC chromatograms and MS characterization of the RibK assays. EIC 1: red= NRF, pink = NFMN, green = AFMN; **EIC 2:** red = NRF pink = NFMN, blue = ARF, green = AFMN; **EIC 3:** red = NFMN, blue = AFMN for 6- amino-riboflavin. **MS1:** ARF: **MS2:** AFMN.

In **figure 13**, EIC 3 displays NFMN and AFMN as the red and blue trace, respectfully, where the AFMN peak is shifted to a higher retention time. This shift is characteristic for all species that undergo spontaneous nitro to amino conversion including any flavin based degradation products.

The spontaneous reduction of 6-nitroflavin analogues to the 6-aminoflavin analogues created ambiguity to the enzymatic phosphorylation of ARF to AFMN with RibK. It was not known if RibK only phosphorylated NRF to yield NFMN, which would then undergo spontaneous reduction to AFMN prior to HPLC and LCMS characterization or if ARF was a substrate for RibK to directly provide AFMN. To determine if ARF was a substrate for Ribk the NRF stock was completely converted to ARF with sodium hyposulfite prior to enzymatic phosphorylation. LCMS analysis of the RibK assays, EIC 1 and 2 of **figure 13**, confirmed the absence of the nitro species where the red and pink trace, present in EIC 1, provided no signal in EIC 2. The presence of AFMN, green trace in EIC 2, in the absence of the nitro species confirmed that ARF is indeed a substrate for RibK. However, like NRF, phosphorylation of ARF is slow and incomplete. The assays were conducted at $70^{\circ C}$ for 2-2.5 hours and to provide the adequate conversion while the native substrate, riboflavin, shows full conversion at $70^{\circ C}$ within 30 minutes (not shown).

Once the nitro to amino conversion was thoroughly understood, proper characterization of BluB assays was possible. Enzymatic assays of NFMN with BluB were inconclusive because a significant amount of AFMN was always present because of the need of a reducing agent in order to conduct the full reaction (NFMN assays are not shown). The reduction of NRF to ARF was always conducted prior to enzymatic assays. Approximately 1 mg of NRF was dissolved in phosphate buffer (pH of 7.5) and had a small amount of sodium hyposulfite added. The eppendorf tube was shaken until the light yellow color representative of reduced ARF went a green (oxidized ARF). The UV-Vis spectrum was acquired to confirm the ARF product, (**Figure 14**). The ARF solution was utilized for RibK/BluB coupled enzymatic assays. The coupled assays were not clean assays due to ARF and AFMN spontaneous degradation within a solution

as well as impurities present from the RibK assays. HPLC and LCMS characterization confirm AFMN is indeed a substrate for BluB. The competitive inhibition of DMB production by AFMN is shown in **figure 15**. The DMB std (purple trace) confirmed the peak with a retention time at 17.2 min to

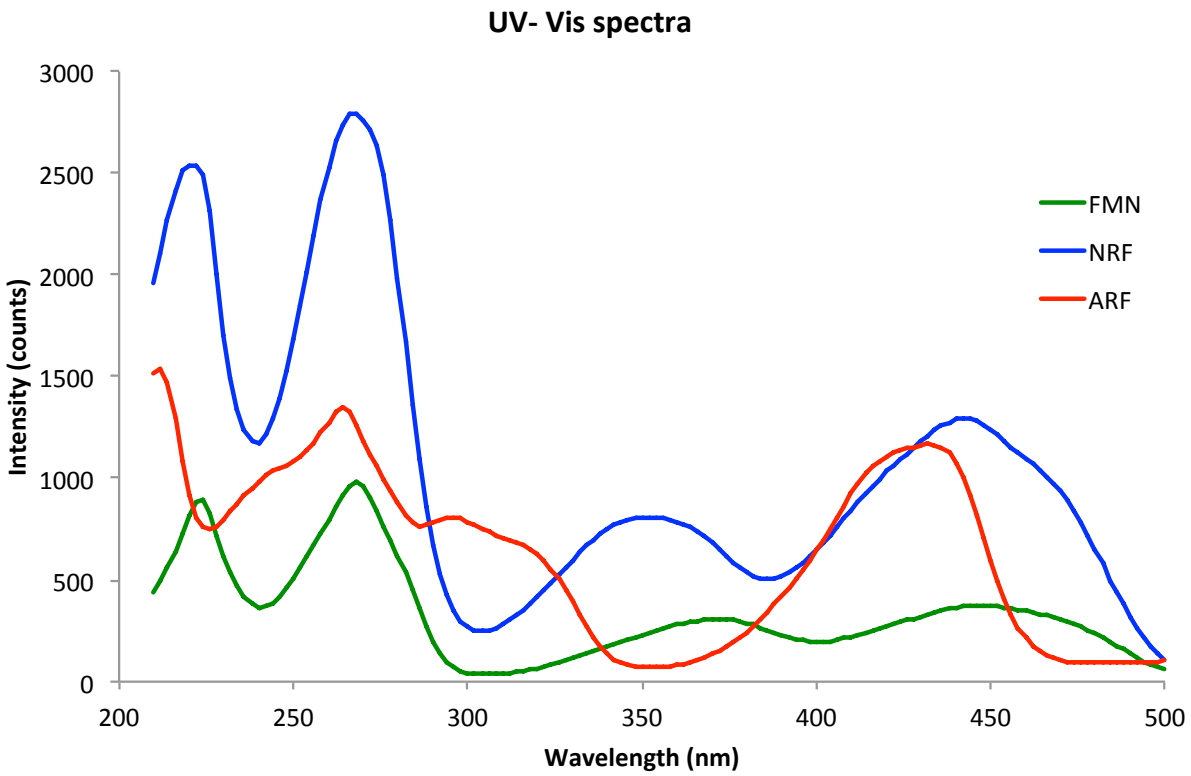


Figure 14. UV-Vis spectrum of FMN, nitro-FMN, and amino-FMN. FMN and nitro-FMN has a λ_{max} at ~ 350 nm while 6-amino-FMN has a λ_{min} at 350 nm. 6-Amino-FMN extinction coefficient is 18000 $M^{-1}cm^{-1}$at 429 nm.[6]

be DMB. This peak is absent in the AFMN full reaction assay (red trace) and present in the FMN full reaction control assay (brown trace). The assays were ran and quenched simultaneously to ensure that peak height and area are indicative of the relative amount present of a species between assays. In the AFMN/FMN full reaction assays less DMB is produced compared to the

FMN full reaction. The decrease in peak area of DMB upon the addition of AFMN was consistently reproduced. This signifies that FMN and AFMN must compete for the same active site; hence, AFMN must bind to the active site of BluB.

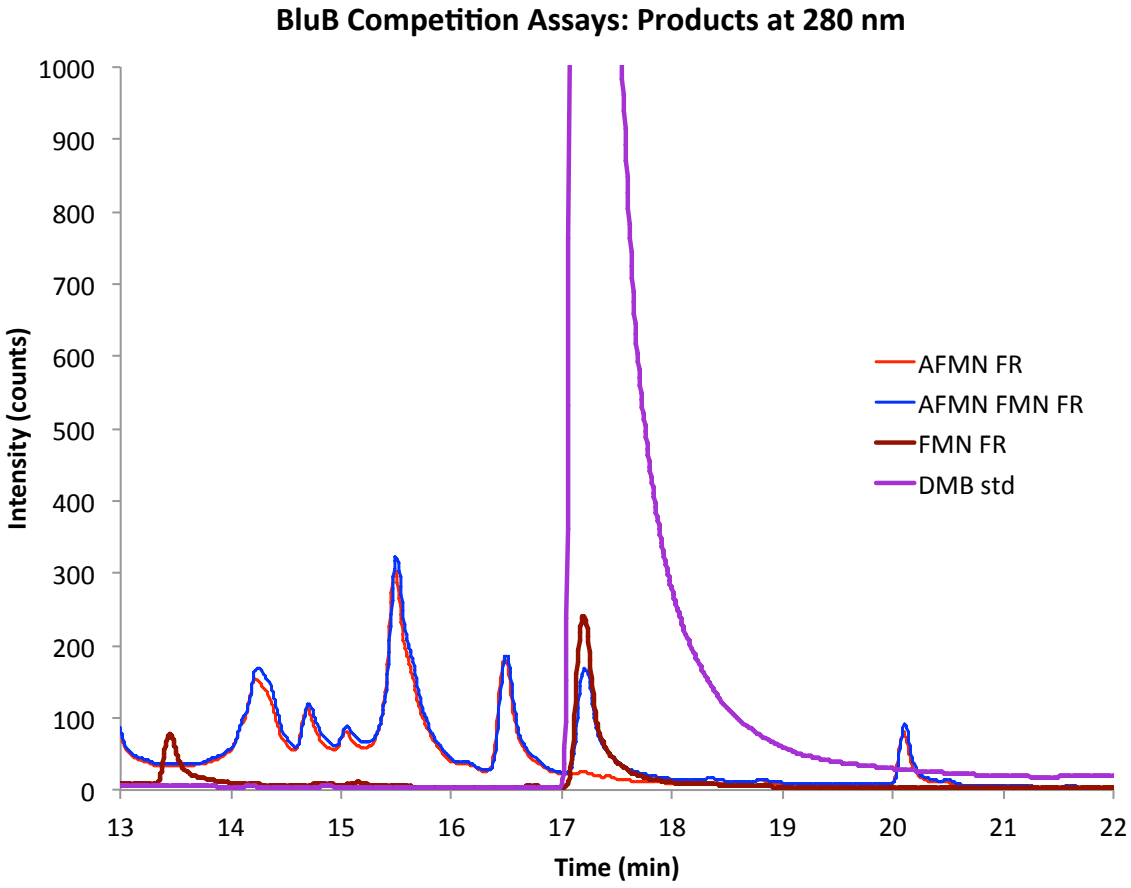


Figure 15. HPLC chromatograms of BluB competition assays: Products at 280 nm. Retention times (t_r): ADMB = 12.2 min, NFMN = 10.0 min, ARF = 12.3 min, AFMN =10.2 min. FR signifies full reaction.

At 450 nm, the consumption of AFMN in the presence of BluB is observed **(Figure 16)**. The consumption of AFMN was inhibited by the presence of FMN while the consumption of FMN was inhibited by the presence of AFMN. The concentration of AFMN was varied to study if the consumption of FMN changed accordingly **(Figure 17)**. The concentration of AFMN was not able to be determined at the time; thus, the standard concentration of AFMN present in the assays was labeled "X". Assays with 2X and 4X relative

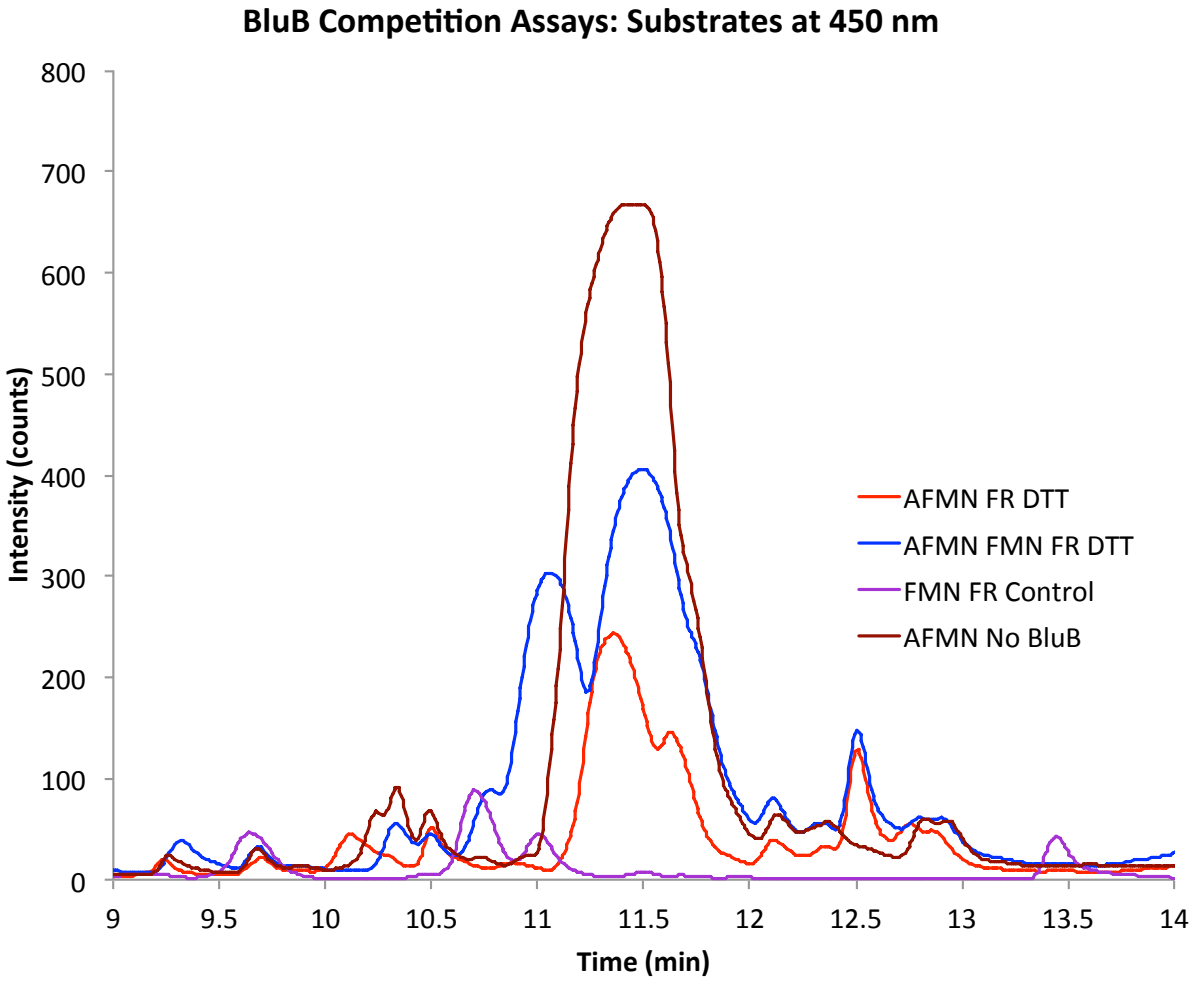


Figure 16. HPLC chromatograms of BluB competition assays: Substrates at 450 nm. Retention times (t_r): FMN = 11.0 min, AFMN = 11.4 min.

concentrations of AFMN were prepared. To do this, the volume of AFMN added from the RibK assay was adjusted and the added volume was compensated by the removal of equal volume of phosphate buffer. This was done to in order to not effect the concentration of other species present, such as FMN.

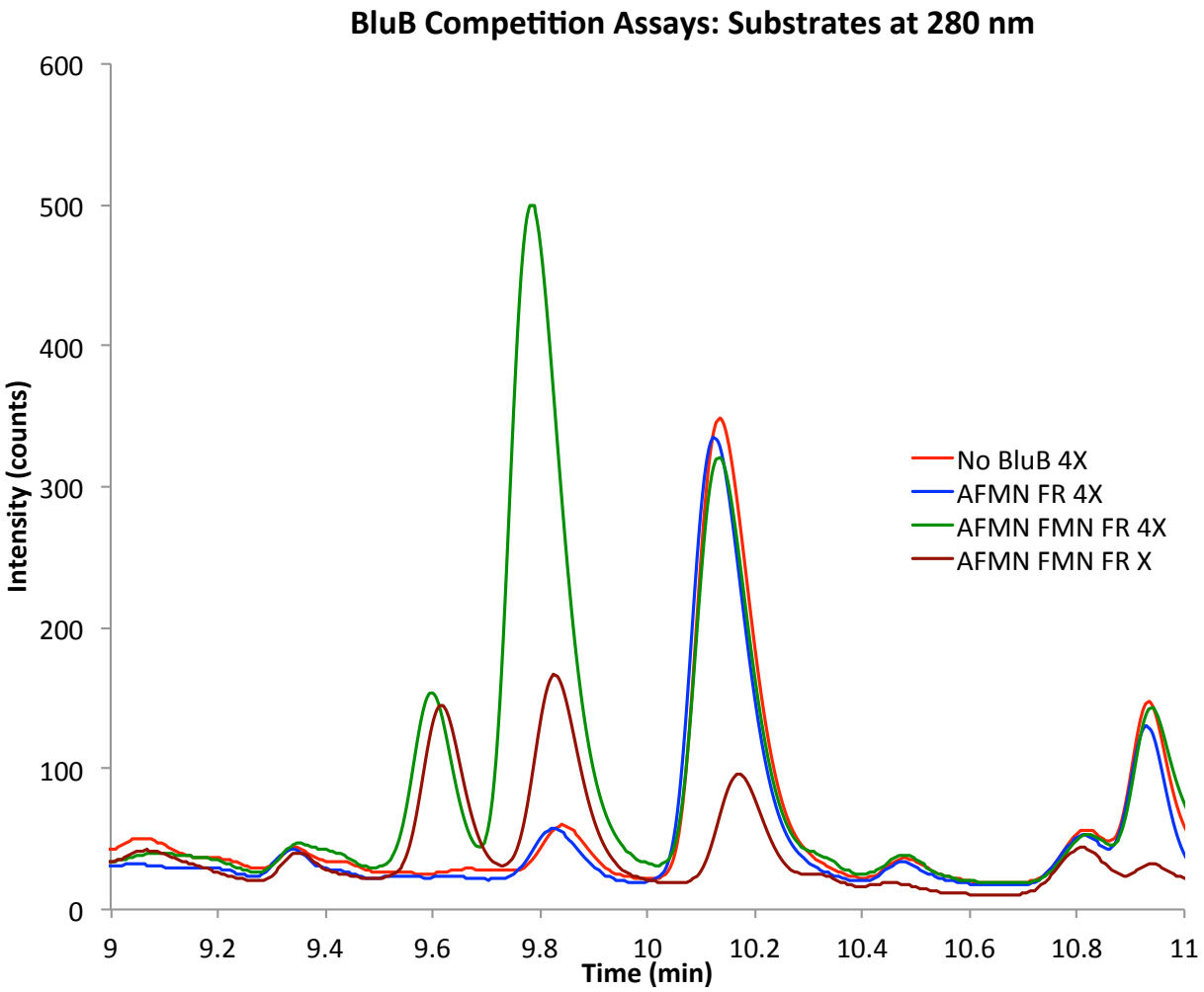


Figure 17. HPLC chromatograms of BluB competition assays: substrates at 280 nm. Retention times (t_r): FMN = 9.8 min and AFMN = 10.15 min. FR signifies full reaction.

These assays confirmed that the presence of AFMN does inhibit the consumption of FMN.

Figure 18 shows the HPLC chromatogram of the products from the assays shown in **figure 17**. The amount of DMB produced decreased from the AFMN FMN FR X assay to the AFMN FMN FR 4X assay. Thus, DMB production is inhibited by AFMN.

Even though these results confirm that AFMN binds to and inhibits BluB enzymatic activity, products from the enzymatic fragmentation of AFMN were not observed in the HPLC chromatograms.

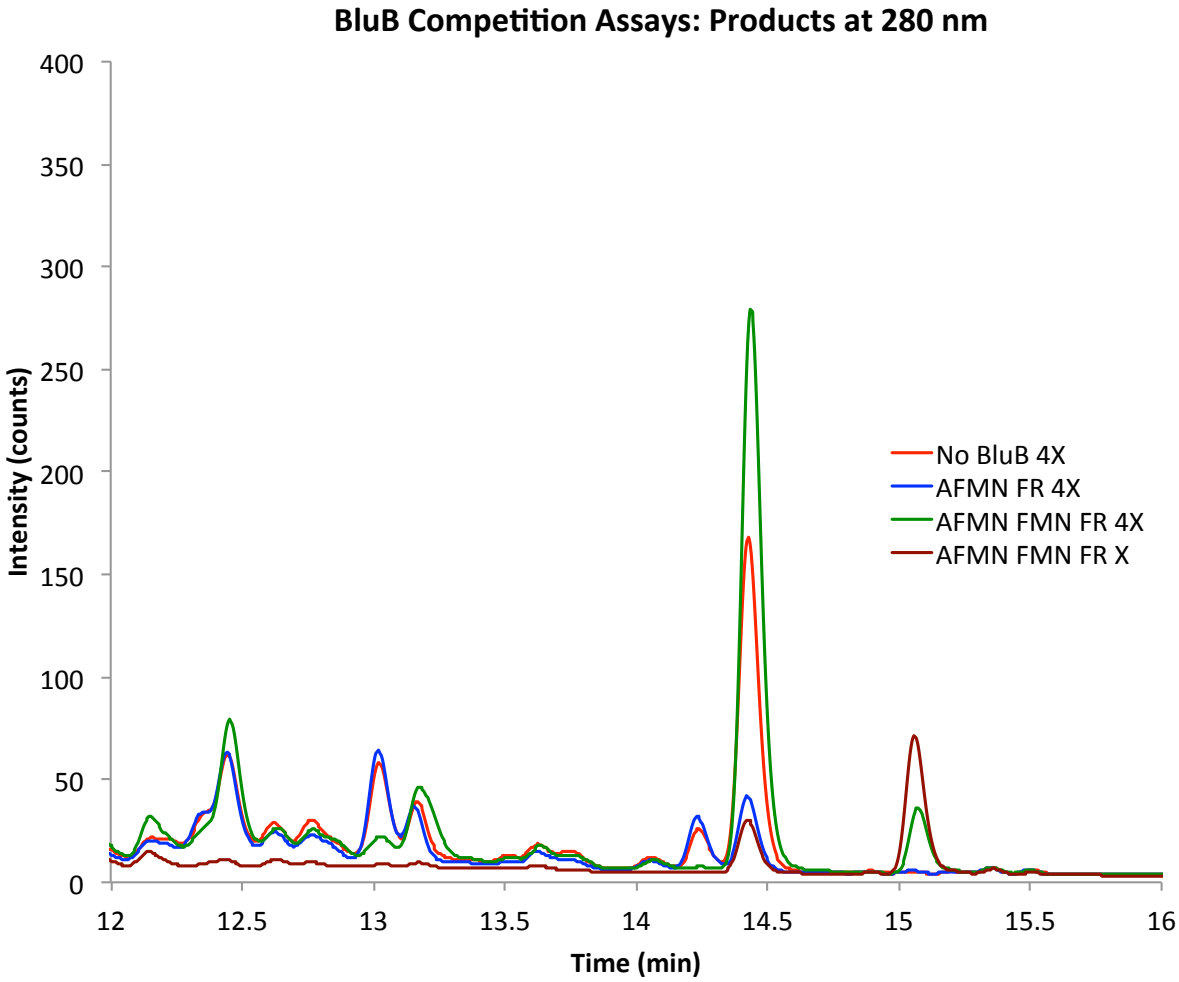


Figure 18. HPLC chromatograms of BluB competition assays: products at 280 nm. Retention times (t_r): DMB = 15.1 min. FR signifies full reaction.

Initially, the reducing agent utilized for the BluB assays was NADH. However, when simultaneous assays were conducted with NADH and DTT as the reducing agent it became apparent that NADH did not reduce AFMN adequately. When the BluB assays were reduced with DTT, AFMN displayed a significant increase in enzymatic activity. The increase in enzymatic activity with DTT allowed for sufficient AFMN product formation for HPLC identification **(Figure 19)**.

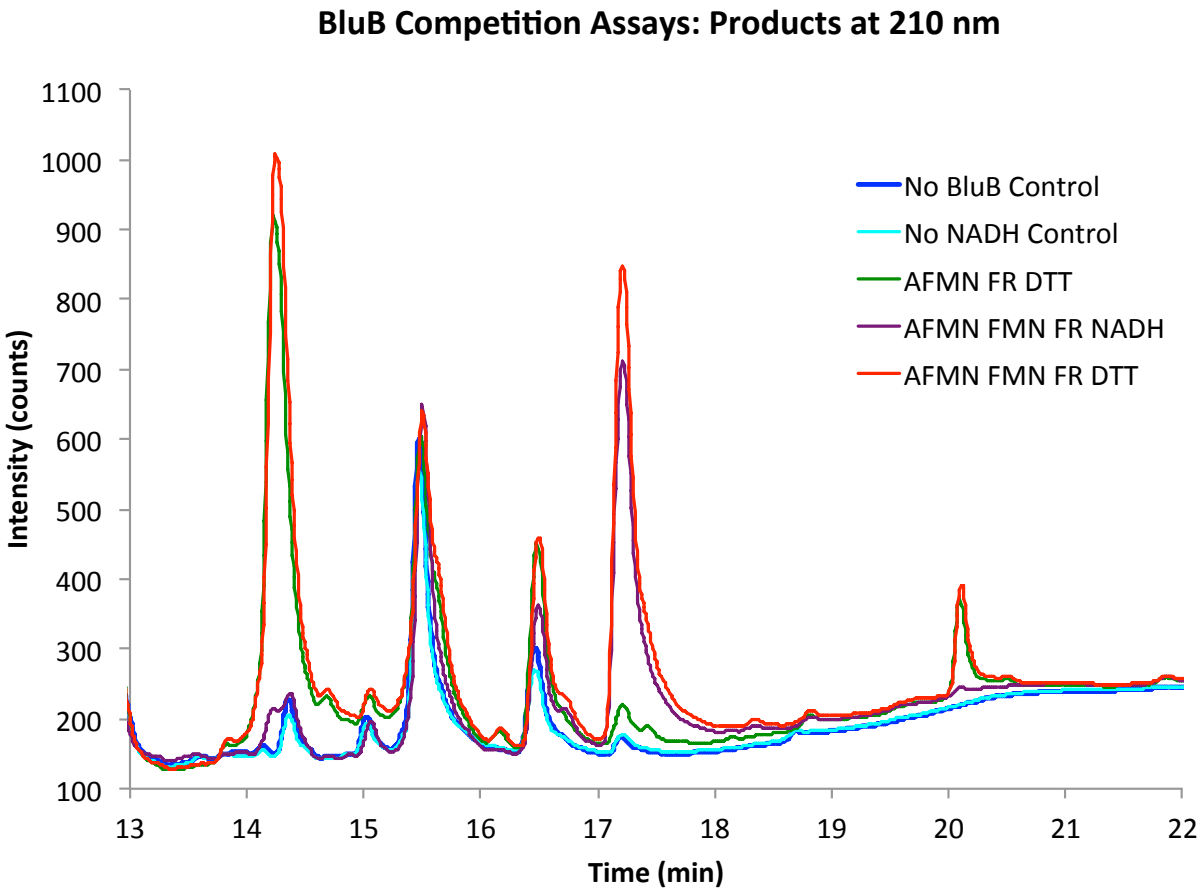


Figure 19. HPLC chromatograms of BluB assays: Products at 210 nm. Retention times (t_r): ADMB = 14.2 min, DMB = 17.2 min. FR signifies full reaction.

AFM products were observed at 210 nm and had retention times of 14.2 min and 20.1 min. These 2 signals were only present in the DTT reduced enzymatic assays and absent in the NADH reduced assays. LCMS characterization of these assays confirmed that amino-DMB (ADMB) was the product of AFMN, **(Figure 20)**. ADMB is believed to be the peak at 14.2 min and the dimer to be the peak at 20.1 min. The peak at 14.2 min, in **figure 19**, is believed to be ADMB because it elutes with a lower retention time with respect to DMB, **(Figure 20)**.

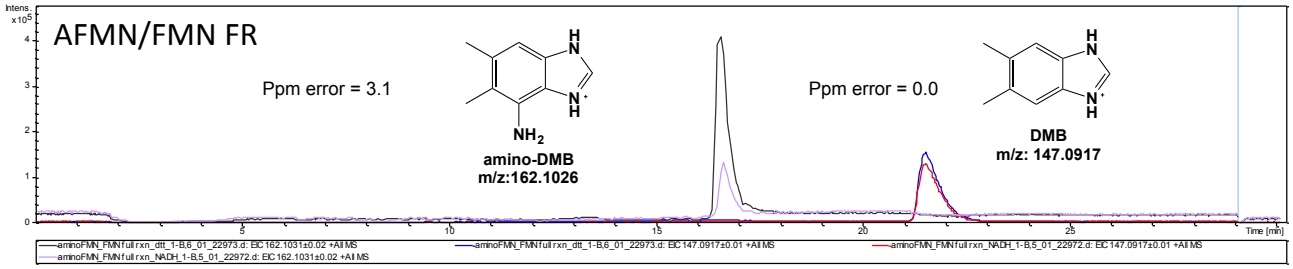


Figure 20. The EIC of ADMB and DMB from the AFMN/FMN competition assays reduced with DTT or NADH: black = ADMB (DTT), purple = ADMB (NADH), blue = DMB (DTT), red = DMB (NADH).

The EIC for ADMB and DMB when reduced with DTT or NADH confirmed that ADMB formation was significantly enhanced when the BluB assays were reduced with DTT rather than NADH, while the production of DMB displayed a small increase with DTT when compared to NADH **(Figure 20)**. The small increase in DMB production when DTT was utilized as the reducing agent may be attributed to the fact that reduced AFMN is a stronger reducing agent than reduced FMN. With DTT, a higher concentration of reduced AFMN is present within the assay and it may act as an additional hydride source for oxidized FMN present within the solution. This can result in a higher activity of reduced FMN present within the assay and cause an

increase in DMB production. The significant increase in the production of ADMB, when reduced with DTT, is attributed to an increase in activity of reduced AFMN within the assay.

Although AFMN has been confirmed to be a substrate analogue for BluB, the amino substituent makes the species hypersensitive to oxidative degradation. This is apparent from how messy the HPLC chromatograms appear. Thus, if a shun product or enzymatic intermediate is formed from AFMN it will most likely degrade rather than accumulate into a distinct peak. Therefore, a method to trap and identify a shun product or an intermediate released by BluB is of high interest. Dansyl Chloride was chosen as the trapping agent. BluB activity assays were conducted with dansyl chloride to characterize any perturbations introduced by the addition of dansyl chloride. BluB assays with its native substrate were conducted in the absence and presence of dansyl chloride. Assays were also conducted with the addition of dansyl chloride after the enzymatic assays were quenched **(Figure 21)**. Dansyl chloride reduces the enzymatic activity of BluB when present during catalysis but does not inactivate the enzyme. Although these assays need to repeated and further characterized, dansyl chloride appears to be a promising trapping reagent because it does not denature or inactivate the protein and can be present during catalysis to trap any intermediate or shun product formed.

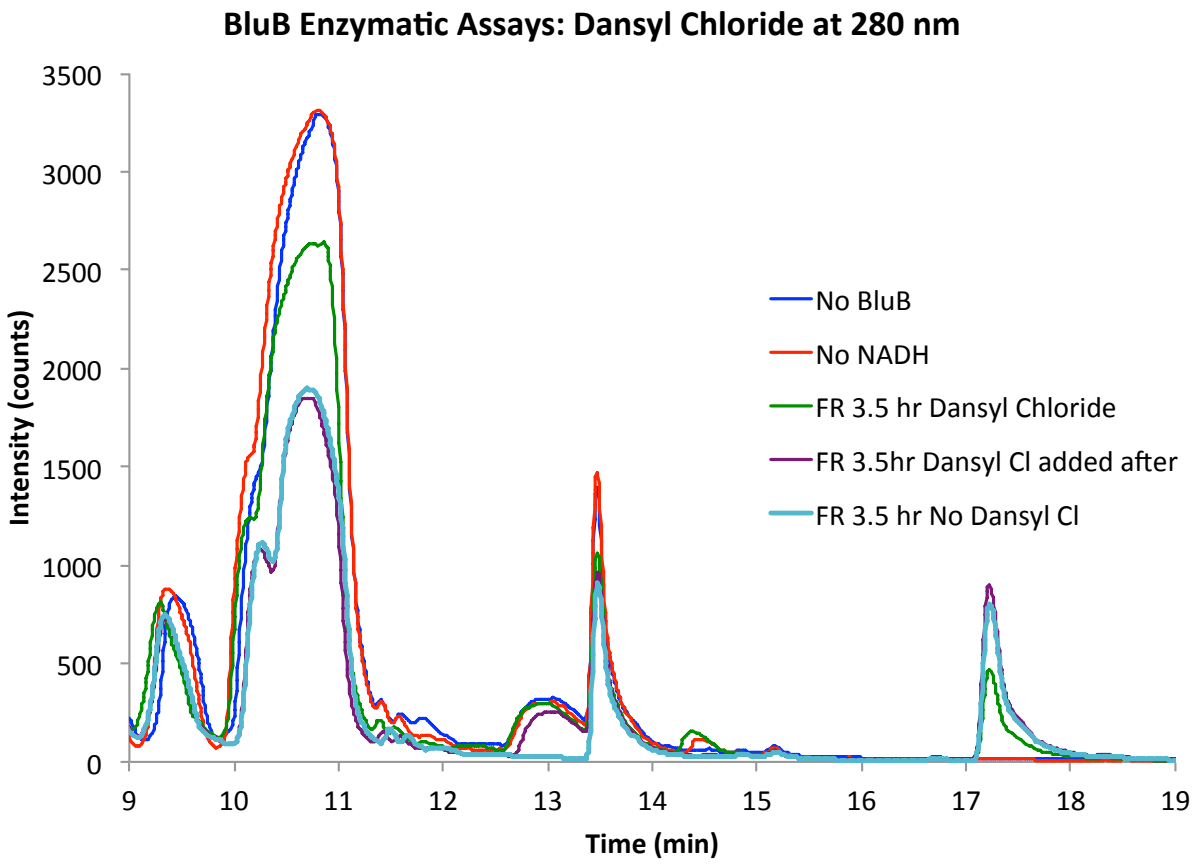


Figure 21. HPLC chromatograms of BluB assays in the presence of dansyl chloride: Products at 210 nm. Retention times (t_r): FMN = 10.7 min, DMB = 17.2 min.

CHAPTER IV

CONCLUSION

To synthesize 6-nitro-FMN, 6-nitro-riboflavin was synthesized and characterized via ^{1}H NMR and UV Vis spectroscopy, and HPLC-MS. Spontaneous reduction into 6-amino-riboflavin was observed via UV Vis spectroscopy, HPLC-MS. Both 6-nitro-riboflavin and 6-amino-riboflavin were enzymatically phosphorylated with riboflavin kinase to yield 6-nitro-FMN and 6-amino-FMN. The addition of reducing agents (NADH and DTT) to the enzymatic assays converted 6-nitro-FMN into 6-amino-FMN. Thus, enzymatic assays with pure 6-nitro-FMN were not possible. Riboflavin kinase/BluB coupled assays were conducted to examine the enzymatic activity of 6-amino-FMN. Competition assays with the native substrate, FMN, confirmed that AFMN possessed inhibition activity. Further studies confirmed AFMN to be enzymatically active when reduced by DTT by the presence of amino-DMB. Amino-DMB was characterized via HPLC and LCMS. Further enzymatic assays need to be conducted to detect formation of any additional shun products or intermediate species. Thus, initial enzymatic assays with the trapping agent, dansyl chloride, have been conducted. In the presence of dansyl chloride, BluB appears to be sufficiently active. Further characterization is needed in order to properly conduct enzymatic assays with AFMN in the presence of dansyl chloride.

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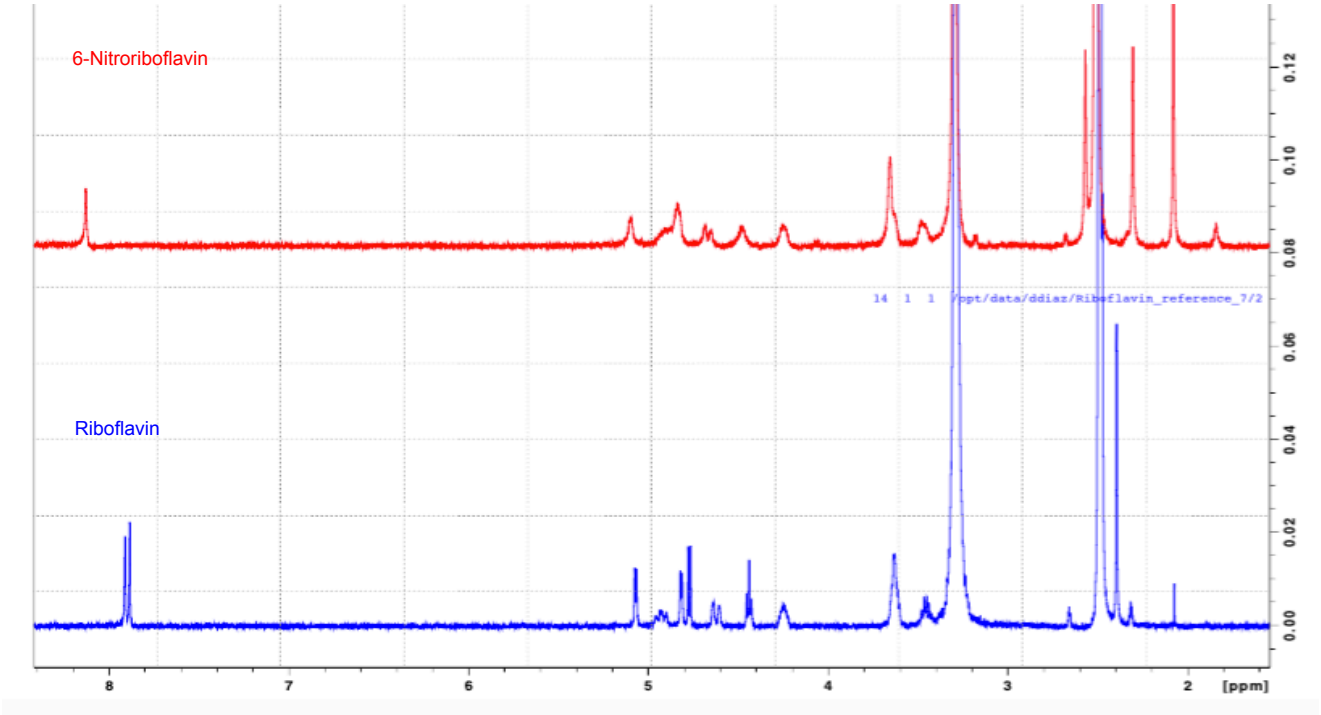
APPENDIX A



Appendix 1. **6-nitroriboflavin and riboflavin ^{1}H spectrum superimposed. 6-nitroriboflavin ^{1}H spectrum: (400 MHz, DMSO) δ** 8.15 (s, 1H), 5.11 (s, 1H), 4.89 (m, 3H), 4.68 (d, 1H), 4.49 (s,1H), 4.26 (m, 1H), 3.66 (s, 3H), 3.47 (m, 1H), 3.33 (s, water), 2.57 (s, 3H), 2.50 (q, DMSO), 2.30 (s, 3H), 2.08 ppm (s, acetone); **Riboflavin ^{1}H spectrum: (400 MHz, DMSO) δ** 7.91 (s, 1H), 7.89 (s, 1H), (s, 1H), 5.07 (s, 1H), 4.93 (t, 1H), 4.81 (s, 1H), 4.77 (s, 1H), 4.62 (d, 1H), 4.44 (t,1H), 4.24 (m, 1H), 3.63 (s, 3H), 3.45 (m, 1H), 3.29 (s, water), 2.50 (q, DMSO), 2.47 (s, 3H 2.39), (s, 3H), 2.08 ppm (s, acetone).